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Permeability of Phospholipid Vesicles to the Tumor Antigen Epitope gp100$_{280–288}$

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Peptides derived from antigenic proteins can be used to generate or expand cytotoxic T lymphocytes (CTLs) that target cells infected by virus or intracellular bacteria, or which are undergoing neoplastic transformation.[1] While the simplest, risk-free administration route for exogenous peptides is intradermal injection, they can also be used for ex vivo pulsing of antigen-presenting cells (APCs) such as dendritic cells.[2,3] Soluble peptides, however, are considered poor immunogens; among the multiple factors possibly involved, enzyme hydrolysis appears to be particularly important. Indeed, fibroblasts,[4] APCs,[5] and dendritic cells[6,7] are reported to express membrane-associated and soluble proteases able to rapidly hydrolyze class I-restricted epitopes. Lipid vesicles, frequently composed of mixed phospholipids and containing cholesterol and/or polymer-bound lipids, have long been used as vehicles for the delivery of drugs, proteins, peptides, and nucleic acids.[8–10] Although the data available on the induction of CTLs by vesicle-carried antigens in humans[11] are scarce, their use has been described extensively in animal models.[12,13] In such applications, peptides encapsulated into lipid vesicles are supposedly shielded from the activity of proteolytic enzymes. Lipid vesicles, however, are permeable to substances of relatively small molecular size,[14,15] and cannot be assumed to be fully impervious to peptides such as the nonameric and decameric class I-restricted epitopes. Among the factors controlling vesicle permeability, a relevant role is played by the size and polarity of the substance carried.[16,17] Permeability is also modified by cholesterol[18] and lipid-bound polymers.[19] Although formation of transient defects of the lipid layer[14] may control the diffusion of small charged species or the flux of species restricted by their interactions with the phospholipid aliphatic chains,[15,20] these mechanisms are unlikely to be meaningful in controlling vesicle permeability toward class I-restricted epitopes.[15] Herein we verified the efflux of class I-restricted epitopes from sonicated vesicles composed of a single phospholipid, and the effect of this parameter on the protection from enzyme hydrolysis provided by the vesicles. The peptide used as model was a tumor antigen epitope, the class I-restricted nonapeptide gp100$_{280–288}$ (Tyr-Leu-Glu-Pro-Gly-Pro-Val-Thr-Ala[21]), while cultured human fibroblasts were used as a source of enzymes.[4]

The permeability of lipid vesicles to gp100$_{280–288}$ was verified by measuring the release of peptide by sonicated phospholipid vesicles. Since results might be affected by possible vesicle instability, measurements were made at low ionic strength (10 mM sodium phosphate, pH 7.2) using charged (distearoylphosphatidylserine) vesicles, and verified with noncharged (distearoylphosphatidylcholine) vesicles, as well as with vesicles prepared with distearoylphosphatidic acid. Vesicles containing gp100$_{280–288}$ were prepared as described in the Experimental Section ("Lipid vesicles"). Measured aliquots of dialyzed vesicles were transferred into dialysis bags, which were in turn transferred to glass test tubes containing phosphate-buffered saline (PBS: 140 mM NaCl, 10 mM sodium phosphate buffer); aliquots were drawn at timed intervals, and peptide concentration was estimated as described below under "Assays".

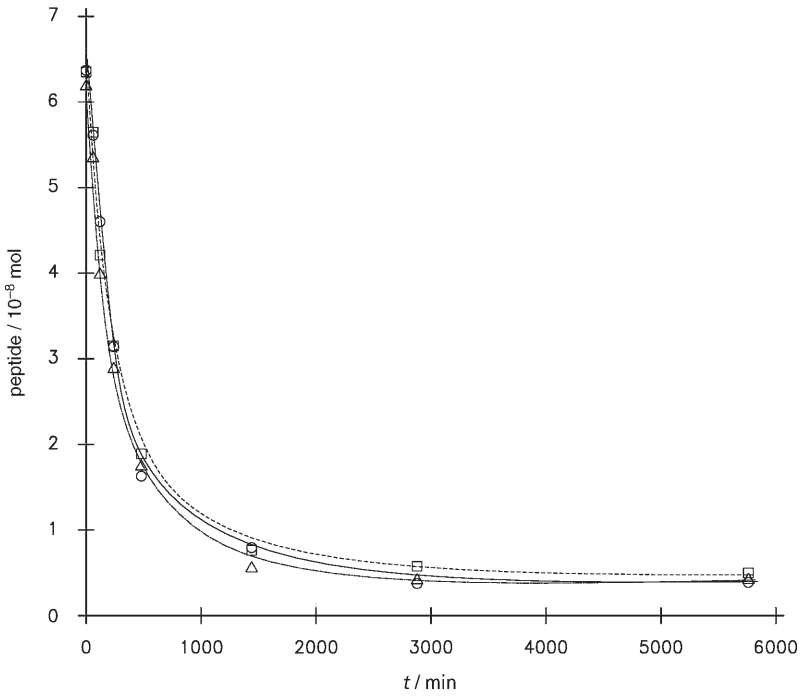

Figure 1. Amount of tritiated gp100$_{280–288}$ contained within the lipid vesicles as a function of dialysis time: distearoylphosphatidic acid (——○——), distearoylphosphatidylserine (----□----), and distearoylphosphatidylcholine (–•–•△–•–•). Symbols indicate experimental points.

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The amount of $gp100_{280-288}$ within the dialysis bag (the vesicle-contained peptide plus the free peptide contained in the bag) as a function of dialysis time is shown in Figure 1. For all three lipids used, these data indicate a time-related decrease of tritium-associated DPM, that is, a reduction in the amount of $gp100_{280-288}$ within the dialysis bag. The curves in Figure 1 indicate that the decrease was rather steep in the first 500 min, flexed near 600 min, and became asymptotic from approximately 3000 min onward. At 5760 min (96 h), the longest dialysis time for all three lipids, an average of 4.35×10^{-9} mol of peptide was found within the vesicles, that is, 6.8% of the value measured at $t=0$ (6.9, 6.1, and 6.4% for phosphatidylserine, phosphatidic acid phosphatidylcholine, respectively). According to the data shown, the average apparent initial velocity of passage was 1.28×10^{-10} mol min^{-1} (1.17×10^{-10}, 1.40×10^{-10}, and 1.27×10^{-10} mol min^{-1} for phosphatidic acid, phosphatidylserine and phosphatidylcholine, respectively). Measured $t_{1/2}$ was 242 min for phosphatidylserine, 234 min for phosphatidic acid, and 170 min for phosphatidylcholine (average $t_{1/2}=220$ min). Besides a relatively high velocity of transmembrane passage (although considerably lower than that measured with species of smaller molecular size,[18]) these data appear to indicate a very limited effect of the lipid charge on the permeability of lipid vesicles to the model epitope.

To evaluate the actual relevance of the phenomenon with respect to the protection toward enzyme hydrolysis, the possible degradation of vesicle-bound $gp100_{280-288}$ was measured by allowing the naked peptide and peptide-containing vesicles to stand in the presence of diploid cultured human fibroblasts[4] cultured as described below under "Cells". Reactions were performed in the presence of confluent cells, and substrate hydrolysis was estimated by measuring the relative amount of intact $gp100_{280-288}$ and of its fragments by reversed-phase chromatography as described elsewhere.[5] Fractions (30 s) were collected and counted for tritium. In accord with previous data,[4] the ultraviolet (Figure 2, insert) and radioactive (Figure 2, main panel) profiles obtained from the reversed-phase column were characterized by corresponding features that underwent time-related variations. These data indicate a time-dependent decrease of the intact nonapeptide (the species eluted at $K'=11.86$), accompanied by an increase of the free $gp100_{280-288}$ -constituent amino acids (the species eluted between $K'=0.70$ and $K'=2.70$), reported to be the main hydrolysis by-product of $gp100_{280-288}$ in the presence of fibroblasts.[5] The formation of free amino acids was accompanied by the appearance of other peaks, the most evident at $K'=7.23$ and $K'=9.34$ and corresponding to the larger fragments derived from $gp100_{280-288}$ hydrolysis.[5] In the case of the naked $gp100_{280-288}$, a plot of the radiolabel corresponding to the intact peptide against reaction time (Figure 3) indicates a time-related decrease in the amount of intact $gp100_{280-288}$, which was accompanied by an increase in the $gp100_{280-288}$-component free amino acids; these data indicate a rapid hydrolysis of the substrate (apparent initial velocity (V_i) $=2.67\times10^{-8}$ mol min^{-1}), 85.4% of which was degraded at the maximum reaction time used (30 min), mostly (over 80%) generating free amino acids. The data for the vesicle-contained peptide were similar: although substrate hydrolysis was perceptibly slower than that of the free substrate (apparent $V_i=1.06\times10^{-8}$ mol min^{-1}), and hydrolysis was incomplete (52.5% of the substrate was hydrolyzed at 30 min), these data indicate that vesicle-contained $gp100_{280-288}$ was only partially protected from the activity of fibroblast-expressed proteases.

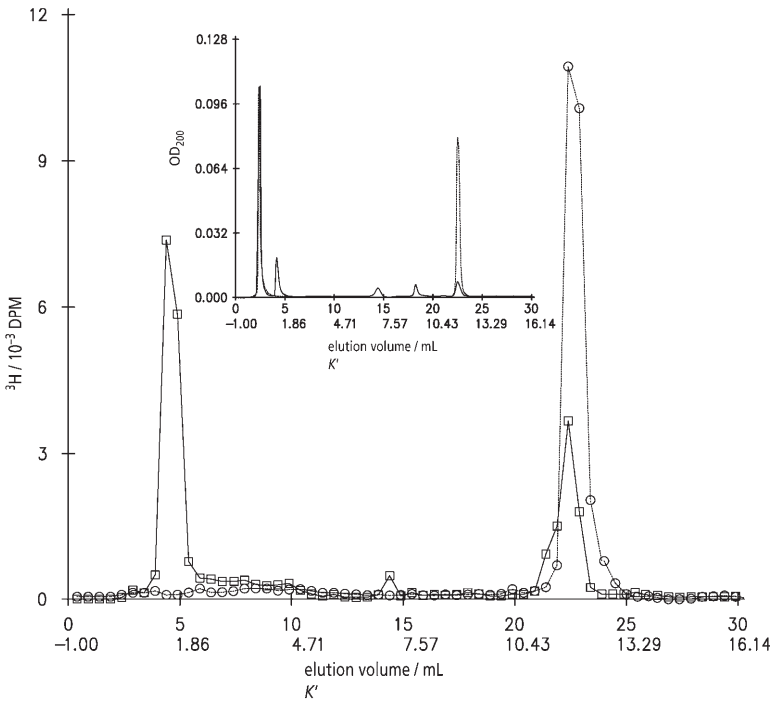

Figure 2. Reversed-phase separation of free $gp100_{280-288}$ and of its hydrolysis by-products after incubation in the presence of cultured fibroblasts. The main panel represents radiolabel at $t=0$ min (-----○-----) and at $t=30$ min (——□——). The inset shows UV profiles at $\lambda=200$ nm for $t=0$ min (-----) and $t=30$ min (——).

Our data indicate a relatively fast (average $t_{1/2}=220$ min) release of $gp100_{280-288}$ from lipid vesicles. These data also indicate that after 70 h of vesicle storage at 24 °C, the model epitope was partially degraded when incubated in the presence of human fibroblasts. According to the above-reported data, after 70 h the vesicle-bound and free peptide were already at equilibrium. Since technical reasons prevented the use of identical experimental setups, the two sets of data are not directly comparable. However, the ratio between the free and vesicle-bound molar fractions at equilibrium indicates that an average of 6.8% of the peptide

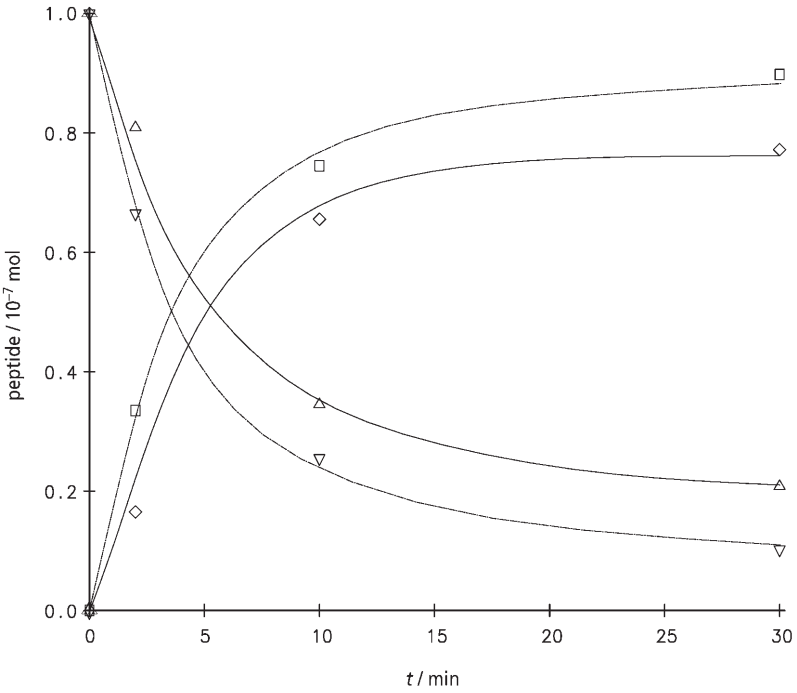

Figure 3. Disappearance of tritiated free and vesicle-contained $gp100_{280-288}$ and appearance of its labeled hydrolysis by-products as function of reaction time in the presence of cultured fibroblasts. Dialyzed $gp100_{280-288}$–containing vesicles were stored 70 h at 24 °C before incubation in the presence of cells. Free peptide: (——), vesicle-contained peptide: (–•–•), intact $gp100_{280-288}$: (△, ▽), and total hydrolysis by-products: (◇, □). Symbols indicate experimental points.

was contained within the vesicles. On the basis of the hydrolysis data, 20.8% of the peptide was intact at 30 min, a figure which fits well with the 17.0% representing the sum of the vesicle-contained plus non-hydrolyzed peptide (10.2% from the naked peptide data) after 30 min in the presence of cells. Thus, membrane permeability appears to be sufficient to explain the amount of peptide hydrolyzed. Since the time elapsed before vesicles intended for in vivo applications are used inevitably exceeds the time necessary to reach equilibrium, the data shown imply that the amount of peptide actually administered is lower than its theoretical amount. In addition, the practice of removing the unincorporated substance by dialysis or chromatography appears to reduce its total amount without providing further advantages.

The data reported herein were obtained with a specific experimental setup; thus, they should, in principle, only apply to species similar to those used in this work. As far as the lipid is concerned, a number of parameters[27] affects vesicle permeability to peptides: notably, increasing the aliphatic chain length slightly reduces permeability;[28] the presence of cholesterol decreases membrane permeability[18] (but see reference [29]), whereas the presence of polymer-conjugated lipids should increase this parameter,[19] although opposite results have also been reported.[30] In addition, a number of other parameters, such as lipid concentration, sonication time, and even the shape of the preparation vial[23] affects vesicle size, size distribution, and the number of lipid layers, modifying permeability.[23] Finally, because of the size distribution of the vesicles used herein (see below under "Steric exclusion chromatography"), and the relatively modest influence of this parameter on permeability,[31] besides the differences induced by geometrical considerations, vesicle size seems unlikely to significantly modify $gp100_{280-288}$ retention. With regard to the peptide, permeability is affected by lipophilicity, size, and conformation.[32,33] For class I-restricted epitopes the size and the relative importance of the charged terminals should imply relatively low permeability.[16,17] However, the generally low polarity of these peptides may imply an high partition coefficient with lipid, which may hamper the peptide release from the lipid. Therefore, as noted by Goren et al,[23] the role of this parameter is a priori unpredictable. Fluorescence anisotropy data (not shown) indicate strong binding between $gp100_{280-288}$ and phosphatidylserine vesicles; thus, this datum may partially explain the relatively fast transmembrane passage measured. It has also been hypothesized[28] that zwitterionic substances are translocated by partition-independent mechanisms, either bilayer transient defects[14,34] or through their neutral form, which is at (left-shifted) equilibrium with the zwitterionic form.[35] However, a nonapeptide should be too large to be translocated by these mechanisms,[17] whereas the negative charge of $gp100_{280-288}$ Glu 3 might conflict with the latter mechanism. Thus, translocation should principally be driven by the lipid partition coefficient. Under these conditions, the membrane permeability to the relatively hydrophilic $gp100_{280-288}$ should be placed at the low end of the possible variations for the generally scarcely hydrophilic class I-restricted epitopes. Although this interpretation is supported by the increase of vesicle permeability to $gp100_{280-288}$ with increased temperature (not shown), irregularities noticeable in the temperature dependence of permeability seem to suggest the involvement of multiple factors.

Despite its apparent simplicity, the permeability of phospholipid vesicles to peptides is a rather complex phenomenon that depends on a number of parameters.[18,27] Thus, it appears wise to assume that vesicle permeability to class I-restricted epitopes is peptide- and lipid-specific, and that both the lipid and the peptide side must be taken into account. However, it is also possible to speculate that modifications of these factors may have a limited impact on the use of peptidic epitopes as vaccines. Indeed, the combination of relatively fast efflux reported herein and rapid in vivo enzyme degradation[4,6] suggests that the optimization of vesicle composition to increase uptake by antigen-presenting cells[36–38]—thus minimizing the amount of peptide leaked from the vesicles before their

uptake—could be a key factor for the successful use of vesicles as vaccine carriers.

Experimental Section

Lipid vesicles: The appropriate amount of a $CHCl_3$ solution of the phospholipid was transferred to a glass test tube, and the volume brought to 1.0 mL with pure $CHCl_3$. A lipid film was created on the test tube walls by evaporating the $CHCl_3$ under reduced pressure (70 °C in a rotating evaporator); residual $CHCl_3$ was removed by maintaining the lipid film under reduced pressure for 360 min at room temperature.[22] A solution of $gp100_{280-288}$ (1 mL, 1×10^{-4} M in 10 mM sodium phosphate, pH 7.2) was then transferred to the lipid-containing test tube. The test tube was vortexed for 10 min and sonicated for 25 min in an ultrasonic bath above the lipid transition temperature. This process produced mostly unilamellar and bilamellar vesicles,[23] the distribution of which was determined by steric exclusion chromatography as described below. To avoid the permeability changes associated with phase transition[16] and vesicle fusion,[24] from this point onward the vesicles were maintained below the lipid transition temperature.

Cells: Confluent diploid human fibroblasts, obtained and maintained as described elsewhere,[5] were used between the 9th and 11th passage. Cell-cycle analysis[25] showed that over 80% of the confluent cells were in G_0 phase.

Hydrolysis: The possible peptide hydrolysis in the presence of fibroblasts was measured as follows: confluent cells were washed twice with PBS (pH 7.2), and the cell layer was covered with a minimal amount of PBS (1 mL for 25 cm^2 flasks). Flasks were transferred to a shaking water bath kept at 37 °C; the temperature was allowed to equilibrate, and intact nonapeptide (1.0×10^{-7} mol) plus tritiated peptide (5.5×10^{-11} mol, 1.51×10^{-7} Ci) were added to the PBS film covering the cells. Reactions were allowed to proceed for the periods of time shown in the Figures, and were stopped by upturning the flasks. The liquid was collected, filtered through 0.45 μm membrane (Sartorius AG, Göttingen, Germany), dried in a rotating evaporator, and stored at −20 °C for analysis as described below.

Assays:*Steric exclusion chromatography*: The size distribution of the lipid vesicles was analyzed by steric exclusion chromatography. Vesicles, prepared in the absence of peptide, were loaded on a TSKG4000 column (7.6×300 mm, 7 μm) equilibrated in PBS. The column was eluted at 1.0 mL min^{-1}, and the effluent was monitored at 300 nm for scattering. The elution characteristics of 69% of the vesicles are consistent with small unilamellar vesicles, whereas the remaining 31%—eluted immediately after the column void volume—can be assigned to larger, possibly multilamellar, vesicles.

Separation of substrate fragments: $gp100_{280-288}$ fragments were separated from each other and from the parent peptide by reversed-phase chromatography. Dried samples, resuspended in the starting mobile phase, were injected into a Lichrospher C-18 (4.6×250 mm, 5 μm; Merck, Darmstadt, Germany), equilibrated in sodium phosphate (1 mM, pH 6.8) + 2% of 80:20 CH_3CN/isopropyl alcohol, and eluted at 40 °C under the conditions described elsewhere.[5] The column was monitored by absorbance at 200 nm. Fractions were collected every 30 s and analyzed as described below. The following standards were eluted at the indicated K' values: the two N- and C-terminal dipeptides Tyr-Leu and Thr-Ala at $K'=9.29$ and 0.33, respectively; the intact nonapeptide at $K'=11.86$; the octapeptide Leu-Glu-Pro-Gly-Pro-Val-Thr-Ala at $K'=9.56$; and Leu-Glu-Pro-Gly-Pro-Val-Thr at $K'=8.92$; $gp100_{280-288}$ constituent amino acids were eluted between $K'=0.70$ and $K'=2.70$.

Peptide permeation across the lipid membrane: Lipid vesicles were prepared as described above in the presence of 1×10^{-3} to 1×10^{-4} M $gp100_{280-288}$ plus tritiated peptide (1.65×10^{-11} mol, 1×10^{5} DPM). Vesicles (950 μL) were then dialyzed for 120 min against 100 mL sodium phosphate (10 mM, pH 7.2). Aliquots of the inner and outer content were withdrawn for scintillation counting, and dialysis bags were transferred to 13×100 mm test tubes containing 10 mL of the above buffer, circulating at 5 mL min^{-1} by a peristaltic pump. Test tubes were maintained at 24 °C (between 24 and 45 °C for temperature assay), and duplicate aliquots of the inner and outer sample were drawn after the time intervals indicated in the Figures. Peptide concentrations were estimated as radiolabel and after reaction with fluorescamine.[26]

Amino acid analysis: Except in the case of free amino acids, amino acid analysis was performed after acid hydrolysis (24 h at 110 °C in 6 N HCl under reduced pressure). Samples were dried on a rotating evaporator and resuspended in phosphate buffer (12.5 mM, pH 7.2). Sample aliquots, plus norvaline used as internal standard, were derivatized with *o*-phthalic aldehyde and analyzed by reversed-phase chromatography (Lichrospher C-18) as described elsewhere.[5] Proline was quantified as the dansyl derivative. Free amino acids in the reversed-phase effluent were quantified as above, omitting acid hydrolysis.

Data analysis: Curves in Figure 1 and 3 were interpolated with a nonlinear iterative procedure (Marquardt–Levenberg), using the equation $y=a\,e^{(bx)}+c\,e^{(dx)}+e$, in which e stands for the base of natural logarithms.

Reagents: The nonapeptide $gp100_{280-288}$ (Tyr-Leu-Glu-Pro-Gly-Pro-Val-Thr-Ala) was synthesized by Neosystem SA (Strasbourg, France). Purity was analyzed by reversed-phase chromatography (C-18) with a slight modification of the technique described above for the separation of substrate fragments. The peptide was tritiated by isotope exchange (Izotop, Budapest, Hungary) obtaining a radiolabel density of 2.76×10^{3} Ci mol^{-1}. Radiochemical purity was assayed by the same reversed-phase column method described above, as well as by thin-layer chromatography on normal phase (Merck Kieselgel 60, eluted at 50 °C in butanol/acetic acid/acetonitrile/water (59.8:10:0.2:30). Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and Sigma (St. Louis, MO, USA). Amino acid and peptide standards were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland) and Serva Feinbiochemica GmbH (Heidelberg, Germany). HCl for peptide hydrolysis and phosphoric acid and NaOH for reversed-phase buffers were Suprapur grade from Merck (Darmstadt, Germany), or an equivalent grade from Romil (Cambridge, UK). Organic modifiers for reversed-phase chromatography were chromatographic grade from Merck or Romil. All other material was of reagent grade and used without further purification.

Keywords: antigens · antitumor agents · drug delivery · lipid vesicles · peptides

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